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Cigarette smoking: profiles of thromboxane- and prostacyclin-derived products in human urine

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Thromboxane (TX) B_2, 2,3-dinor-TXB_2, 11-dehydro-TXB_2, 6-oxoprostaglandin (PG)$F_{1\alpha}$ and 2,3-dinor-6-oxo-$PGF_{1\alpha}$ were measured in 24 h urine samples obtained from 30 apparently healthy chronic cigarette smokers and 37 closely matched non-smoking control subjects. Samples were analysed using a newly developed assay based on immunoaffinity chromatography and capillary column gas chromatography/electron capture negative ion chemical ionisation mass spectrometry. There were significant and comparable increases in the excretion rates of both 2,3-dinor-TXB_2 and 11-dehydro-TXB_2 in the smoking compared with the non-smoking group ($2P < 0.001$). Excretion rates of 2,3-dinor-TXB_2 were 418 ± 35 and 265 ± 26 pg/mg creatinine in the two groups, respectively. 11-Dehydro-TXB_2 excretion rates were 440 ± 54 and 221 ± 18 pg/mg creatinine, respectively (mean ± S.E.). There were significant ($2P < 0.05$) positive correlations between average reported cigarette consumption and excretion of both thromboxane metabolites. There were small but significant ($2P < 0.02$) increases in the excretion rates of both 6-oxo-$PGF_{1\alpha}$ and 2,3-dinor-6-oxo-$PGF_{1\alpha}$ in the smoking compared with the non-smoking group. There was no significant difference in the rates of excretion of TXB_2 in the two groups. The effects of acute cigarette smoke exposure (five cigarettes in 2 h) was also studied in four normally non-smoking healthy volunteers. There was no significant change in the excretion rate of any of the eicosanoids measured during control and smoking periods (at least 2 weeks apart), indicating that increased TXA_2 biosynthesis in chronic smokers is unlikely to be a consequence of acute platelet activation.

Introduction

Thromboxane (TX)A_2 is produced by activated platelets, macrophages and neutrophils. It causes platelet aggregation and is a potent vasoconstrictor [1]. Prostacyclin (PGI_2) is produced by vascular endothelium, is both a potent inhibitor of platelet aggregation and a vasodilator [2]. An alteration in the relative rate of production of these eicosanoids may play a part in the pathophysiology of vascular disease [3–5]. This hypothesis has proved difficult to test, because of the complexity of measuring TXA_2 and PGI_2 production in vivo. The least invasive measures of TXA_2 and PGI_2 production are provided by determination of urinary excretion of their hydrolysis products, TXB_2 and 6-oxo-$PGF_{1\alpha}$ and their respective 2,3-dinor metabolites [6,7]. TXA_2 is also substantially metabolised by an 11-keto reductase pathway to 11-dehydro-TXB_2 which also appears in the urine [8,9]. Determinations of these products as an index of eicosanoid biosynthesis can be arduous. Urine presents a highly complex and variable medium from which prostaglandins and thromboxanes are difficult to purify without multiple chromatographic procedures. This restricts the opportunity for simultaneous analysis of a number of related products in a single sample. Despite such difficulties, it has been possible to demonstrate abnormal TXA_2 and/or PGI_2 production in a number of diseases (reviewed in Refs. 4 and 5).

Cigarette smoking predisposes strongly to vascular disease [10–12], and it has been found that urinary excretion of 2,3-dinor-TXB_2 is increased in smokers [13,14]. Indirect evidence suggests that this increase may be due to platelet activation [14]. However, measure-

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Abbreviations: TX, thromboxane; PG, prostaglandin; PGI_2, prostacyclin.

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ment of a single metabolite leaves open the question as to whether this reflects an increase in TXA_2 biosynthesis or whether it is a consequence of altered metabolism. In this present study, we have developed a simple yet highly selective method based on immunoaffinity chromatography and gas chromatography/electron capture negative ion chemical ionisation mass spectrometry (GC-ECMS) that allows us to profile excretion of TXB_2, 2,3-dinor-TXB_2, 11-dehydro-TXB_2, 6-oxo-$PGF_{1\alpha}$ and 2,3-dinor-6-oxo-$PGF_{1\alpha}$ in human urine. We have applied this to the measurement of these eicosanoids in the urine of chronic smokers and of controls matched closely for age, sex, social class and physical activity. We have further studied the effect of acute exposure to cigarette smokers in normally non-smoking volunteers on urinary eicosanoid metabolite excretion, to determine whether the increase in thromboxane metabolite excretion is due to acute platelet or other cellular activation by absorbed components of smoke.

Materials and Methods

Materials

All solvents and reagents were of analytical grade. Water was purified using a Milli-Q water purification system (Millipore (U.K.) Limited, Harrow, Middlesex, U.K.). Deuterated and non-deuterated prostaglandin and thromboxane standards (excepting [$^2H_4$]11-dehydro-TXB_2) were obtained as gifts from Dr. John Pike, The Upjohn Company, Kalamazoo MI, U.S.A. or were purchased from Cayman Chemicals, Ann Arbor, MI, U.S.A. [^{3}H]6-Oxo-$PGF_{1\alpha}$ and [^{3}H]TXB_2 were obtained from New England Nuclear, Stevenage, U.K. 1-Ethyl-3-(3-diethylaminopropyl), carbodiimide hydrochloride, diisopropylethylamine, bis(trimethylsilyl)trifluoroacetamide, human serum albumin (essential fatty acid free), Freund's complete adjuvant and CNBr-activated Sepharose 4B were obtained from Sigma Chemical Co., Poole, U.K. 3,5-Bistrifluoromethylbenzyl bromide was purchased from Fluorochem, Glossop, Derbyshire, U.K. A Vac Elut system, Bond Elut reservoirs, luer stopcocks, polyethylene frits, and capillary columns for GC analysis were obtained from Jones Chromatography, Hengoed, Mid Glamorgan, U.K.

Synthesis of [$^2H_4$]11-dehydro-TXB_2

[$^2H_4$]11-Dehydro-TXB_2 was prepared by selective oxidation of [$^2H_2$]TXB_2, using moist silver oxide. Silver oxide was freshly prepared by stirring 2 M sodium hydroxide (20 ml) and silver nitrate (340 mg in 5 ml water) at ambient temperature for 10 min. The product was isolated by filtration, washed sequentially with water (50 ml), acetone (50 ml) and diethyl ether (50 ml) and dried under a stream of nitrogen. Silver oxide (2 mg) was added to [$^2H_4$]TXB_2 (100 mg) in chloroform/methanol (300 μl; 50:50, v/v) under N_2 at ambient temperature for 30 min. A further 500 μl of chloroform/methanol was then added and the mixture transferred to a glass Eppendorf centrifuge tube and centrifuged for 1 min. The supernatant was aspirated and the pellet washed with 2×500 μl chloroform/methanol. Supernatants were combined and the pellet discarded. Solvent was removed by evaporation under a stream of nitrogen. The residue was then taken up in ethyl acetate (2×200 μl) and the remaining silver oxide precipitate removed by centrifugation. The supernatant, containing thromboxanes was evaporated to dryness and stored in ethanol at −20°C. Conversion to [$^2H_2$]11-dehydro-TXB_2 was determined by GC-ECMS by comparing with known amounts of non-deuterated internal standards of TXB_2 and 11-dehydro-TXB_2. The product contained 20% [$^2H_4$]11-dehydro-TXB_2 and 80% [$^2H_4$]TXB_2 and was used as a combined internal standard in subsequent experiments without further purification.

Preparation of antibodies

In contrast to radioimmunoassay, immunoaffinity chromatography of prostaglandins and thromboxanes requires a relatively large and constant supply of antibodies of low specificity. Cross-reactivity with metabolites and other structurally related compounds of interest is desirable, since this makes simultaneous extraction and analysis possible. Conjugates of TXB_2 and 6-oxo-$PGF_{1\alpha}$ were prepared by the method of MacLouf [15]. Briefly, TXB_2 or 6-oxo-$PGF_{1\alpha}$ and [^{3}H]TXB_2 or [^{3}H]6-oxo-$PGF_{1\alpha}$ (approx. $2 \cdot 10^6$ dpm) were dissolved in 500 μl acetonitrile/water (95:5, v/v), diluted with water (9 ml) and the pH adjusted to 5.5 using HCl. 1-Ethyl-3-(3-diethylaminopropyl)carbodiimide hydrochloride (29 mg) was added and the mixture allowed to stand at ambient temperature for 1 h. Human serum albumin (20 mg; essential fatty acid free) was then added and the solution was allowed to stand for 2 h at ambient temperature and overnight at 4°C. The solution was then dialysed overnight at 4°C against 3×6 litre water at pH 7.4 to separate unreacted ligand from the conjugate. This procedure resulted in the conjugation of approx. 30 molecules of prostanoid per molecule of albumin.

New Zealand White rabbits were immunised with the conjugate of either 6-oxo-$PGF_{1\alpha}$ or TXB_2 (0.5 mg in 1 ml water per rabbit) emulsified with an equal volume of Freund's complete adjuvant. The emulsion was injected subcutaneously at three sites on the back and intramuscularly at one site in the flank. Booster doses (0.5 mg) were administered similarly at 4 and 8 weeks. Venous blood (20 ml) was collected at 9 weeks and monthly thereafter and serum prepared by incubation in plain glass tubes at 37°C for 1 h and centrifuging at $1000 \times g$ for 10 min. Sera at week 9 contained antibodies that recognised 6-oxo-$PGF_{1\alpha}$ or TXB_2 with 50%

binding at a dilution in the range 1 : 10000–1 : 150000. Further booster doses of conjugate were administered to the rabbits when the antibody titre began to fall.

Cross-reactivities of these antisera with their respective dinor metabolites and of the TXB_2 antiserum with 11-dehydro-TXB_2 were determined by their displacement of [^{3}H]6-oxo-$PGF_{1\alpha}$ or [^{3}H]TXB_2 over a concentration range of 20 pg–2 ng, using a modification of the radioimmunoassay described by MacLouf [16]. These assays were carried out in buffered gelatine saline at pH 4.0, 7.4 and 8.0 to determine the affinity of the antibodies for the ring open and closed isomeric forms of the prostaglandins and thromboxanes. Standards were equilibrated overnight at 4°C in appropriate buffer before study. The affinities of TXB_2 and 6-oxo-$PGF_{1\alpha}$ with their respective antisera were independent of pH. Cross-reacties of TXB_2 antisera with 2,3-dinor-TXB_2 were in the range 55–75% and were also independent of pH. Antisera did not recognise the ring open dicarboxylic acid form of 11-dehydro-TXB_2 but did cross-react (10–14%) with the δ-lactone form predominating at pH 4.0. The ketone and γ-lactone isomers of 2,3-dinor-6-oxo-$PGF_{1\alpha}$ had different affinities for the 6-oxo-$PGF_{1\alpha}$ antisera and cross-reacted in the ranges 100–125 and 15–20%, respectively.

Determination of the time for equilibration of 11-dehydro-TXB_2 with its deuterated internal standard

Since only the δ-lactone isomer of 11-dehydro-TXB_2 cross-reacted with antibodies raised against TXB_1, it was necessary to ensure complete equilibration of endogenous metabolite in urine with any deuterated 11-dehydro-TXB_2 added as internal standard before immunoaffinity extraction. Kumlin and Granström [16] have reported that equilibration between the two isomers is very slow. Urine was collected from five normal drug-free subjects and pooled. 10 ml samples were diluted 1 : 1 (v/v) with buffer at pH 4.0 at 4°C. [$^2H_4$] 11-Dehydro-TXB_2 (2 ng) was added and samples were kept at 4°C and extracted either immediately and at appropriate time intervals up to 7 days. They were derivatised and assayed by GC-MS as described below. Equilibration was complete within 48 h at 4°C.

Preparation of prostanoid immunoaffinity columns

Antibodies were immobilised on Sepharose, using a method based on that of Krause et al. [17]. Briefly, antiserum (2 ml) and saturated ammonium sulphate (1.3 ml) were stirred at room temperature for 30 min. The mixture was centrifuged and the precipitate dissolved in 0.1 M sodium bicarbonate/0.5 M sodium chloride at pH 8.3 (5 ml) and was dialysed overnight against 2 × 3 litre water at 4°C to remove excess ammonium sulphate. CNBr-activated Sepharose 4B (2.4 g) was washed with 500 ml 10^{-3} M HCl using a Buchner filter flask. The Sepharose was then added to the antibody solution and gently mixed for 2 h at ambient temperature. The solid phase was filtered and washed sequentially with 20 ml 0.1 M acetate buffer/1 M NaCl at pH 4.0 followed by 20 ml 0.1 M borate buffer/1 M NaCl at pH 8.0. Any remaining active groups on the Sepharose were inactivated by washing with 20 ml 1 M ethanolamine. After two further washes with acetate and then borate buffer, as described above, the antibody immobilised on Sepharose was stored in 25 ml 0.1 M phosphate buffer (pH 7.4) at 4°C.

Aliquots (0.5 ml) of Sepharose suspension containing antibodies raised to 6-oxo-$PGF_{1\alpha}$ and TXB_2 were combined and placed in polypropylene Bond Elut reservoirs (1.5 ml capacity) equipped with 20 μm porous polyethylene frits, luer stopcocks and stoppers. These columns were stored at 4°C.

Subjects and urine collection

Studies were approved by the Ethics Committee of Hammersmith and Queen Charlottes Hospitals. 24 hour urine samples were collected without preservative from 30 apparently healthy habitual cigarette smoking males (are range 18–39; median 24) and 37 non-smoking males (age range 17–40; median 22) recruited from the Royal Worcester and Sherwood Foresters British army regiment during the same 24 h period. Reported cigarette consumption in 27 subjects ranged from 3–40 per day (median consumption 18), and duration of smoking ranged from 1–26 years (median 11 years). None of the subjects had taken aspirin for a week or more before study, and none had taken any other drugs for at least 3 days before study. The pH of all urine samples was noted.

Acute cigarette exposure was studied using Benson & Hedges filter-tipped cigarettes smoked by four apparently healthy drug-free, normally non-smoking volunteers recruited from the staff of Hammersmith Hospital. Subjects were studied on two occasions at least 2 weeks apart according to a cross-over design. After voiding the bladder, subjects were water-loaded (500 ml followed by 2 × 250 ml at $t = 1$ and 2 h) to allow hourly collection of spontaneously voided urine. After a baseline period of 1 h, subjects smoked one cigarette at the beginning of the second hour. They smoked a further four cigarettes during the third hour. A 24-h urine sample was then collected. Blood (10 ml) was collected from a forearm vein before, 1 and 22 h after the smoking period for determination of cotinine as an index of cigarette consumption. Control urine and blood samples were collected from the same subjects under the same conditions at the same time of day on a separate occasion. Plasma cotinine concentrations were determined by GC-electron impact mass spectrometry as described previously [18].

Extraction of urines for prostanoid analysis

For analysis of 6-oxo-$PGF_{1\alpha}$, TXB_2 and their 2,3-dinor metabolites, urine samples (10 ml) were diluted with

0.1 M phosphate or Tris buffer at pH 8.0 to favour the ketone isomers of prostaglandins, necessary for GC-ECMS. Exact amounts of internal standards approximating to 8 ng [$^2H_4$]TXB_2, 5 ng [$^2H_4$]2,3-dinor-TXB_2, 5 ng [$^2H_4$]6-oxo-$PGF_{1\alpha}$ and 5 ng [$^2H_4$]2,3-dinor-6-oxo-$PGF_{1\alpha}$ were added. The samples were stored at −20 °C until assayed. For analysis of 11-dehydro-TXB_2, urine samples (10 ml) were acidified to pH 4.0, and a mixture containing [$^2H_4$]11-dehydro-TXB_2 (2 ng) and [$^2H_4$]TXB_2 (8 ng) was added. They were stored at −20 °C, but before extraction they were allowed to stand at 4 °C for 48 h to ensure equilibration of internal standard and endogenous 11-dehydro-TXB_2. Samples were centrifuged at 1000 × *g* to remove particulate matter immediately before extraction.

Extraction of urine was carried out using a modification of the method of Chiabrando et al. [19]. Sepharose columns were equipped with reservoirs (20 ml capacity) and washed with 10 ml water, 10 ml acetone then 10 ml water using a Vac Elut system. Urine samples were applied to the columns under vacuum. They were washed with 10 ml water, the stopcock was closed and acetone/water (0.5 ml; 95 : 5, v/v) was added. Columns were stoppered and the Sepharose suspensions containing antibody and bound antigen from the urine were rotated mechanically for 15 min. The acetone/water eluate was collected. A further 2 × 0.5 ml acetone/water was washed through the columns and collected. The combined eluates containing prostaglandins and thromboxanes were taken to dryness under a stream of nitrogen. The columns were washed with acetone and then water and stored for re-use in phosphate buffer at pH 7.4 at 4 °C. Before using for quantitative analysis, newly prepared columns were subjected to this entire extraction procedure to remove traces of TXB_2 bound to the Sepharose, which may originate from the antiserum. Immunoaffinity columns were re-used up to 20, but more usually up to 10 times. Their lifetime is limited by accumulation of particulate material from the urine rather than loss of binding sites.

Derivatisation of prostanoids

Samples were converted to 3,5-bistrifluoromethyl benzyl esters (to provide an electron-capturing group suitable for ECMS), and then to trimethyl silyl ether derivatives. They were dissolved in acetonitrile (50 μl), and 3,5-bistrifluoromethyl benzyl bromide in acetonitrile (33% solution; 10 μl) and diisopropylethylamine (10 μl) were added. Solutions were thoroughly mixed and allowed to stand at ambient temperature for 15–30 min. Solvent and excess reagents were removed under a stream of nitrogen. Bis(trimethylsilyl)trifluoroacetamide (100 μl) was added and the solutions allowed to stand overnight at ambient temperature. Reagent was removed under a stream of dry nitrogen and the residues were reconstituted in *n*-dodecane (10–15 μl) ready for GC-MS analysis. The samples were stored with dessicant at −20 °C.

Gas chromatography-mass spectrometry of eicosanoids

Samples were assayed using a Finnigan 4500 combined gas chromatograph-mass spectrometer operating in the electron capture mode using ammonia as reagent gas. Capillary columns were 30 m DB-1 (0.25 mm i.d.; film thickness 0.25 μm). Prostanoid samples were injected using a Grob injector in the splitless mode at 250 °C and the column was programmed from a temperature of 200 to 325 °C at 20 C°/min. Quantitative analyses were carried out using selected ion monitoring of carboxylate anions at *m/z* 585 for 6-oxo-$PGF_{1\alpha}$ and TXB_2 and *m/z* 589 for their deuterated internal standards. The 2,3-dinor metabolites and deuterated standards were monitored simultaneously at *m/z* 557 and 561, respectively. 11-Dehydro-TXB_2 and deuterated 11-dehydro-TXB_2 were monitored at *m/z* 511 and 515, respectively. Calibration curves were prepared using standards extracted from buffer at appropriate pH in the range 50 pg–5 ng.

Statistical analysis

The best fit for calibration curves for quantitative analysis were obtained using an unweighted least-squares linear regression analysis. Excretion rates of prostanoid metabolites in smokers and non-smokers were distributed normally. Results are expressed as mean ± S.E. Differences were analysed using Student's *t*-test and were considered significant when $2P < 0.05$. Correlations were sought using the method of least-squares linear regression, and considered significant when $2P < 0.05$.

Results

Typical selected ion-monitoring chromatograms, used to quantify TXB_2, 2,3-dinor-TXB_2, 11-dehydro-TXB_2, 6-oxo-$PGF_{1\alpha}$ and 2,3-dinor-6-oxo-$PGF_{1\alpha}$ extracted from urine are illustrated in Fig. 1. Typical calibration curves, prepared for prostaglandin and thromboxane standards extracted from water and from a pooled urine sample are shown in Fig. 2. The intercepts on the ordinate for the standards extracted from urine represent endogenous prostaglandin. The isotopic purity of the deuterated standards is indicated by the intercepts on the ordinates of the curves extracted from water. In all cases the purity was more than 99.95%. There was no evidence of carry over of eicosanoids from one extraction to the next (this would be detected as a high intercept on the ordinate of calibration curves extracted from water). However, we noted that newly prepared columns contain traces of TXB_2. This presumably originates from the serum from which the antibody was obtained and must have been protein bound to be

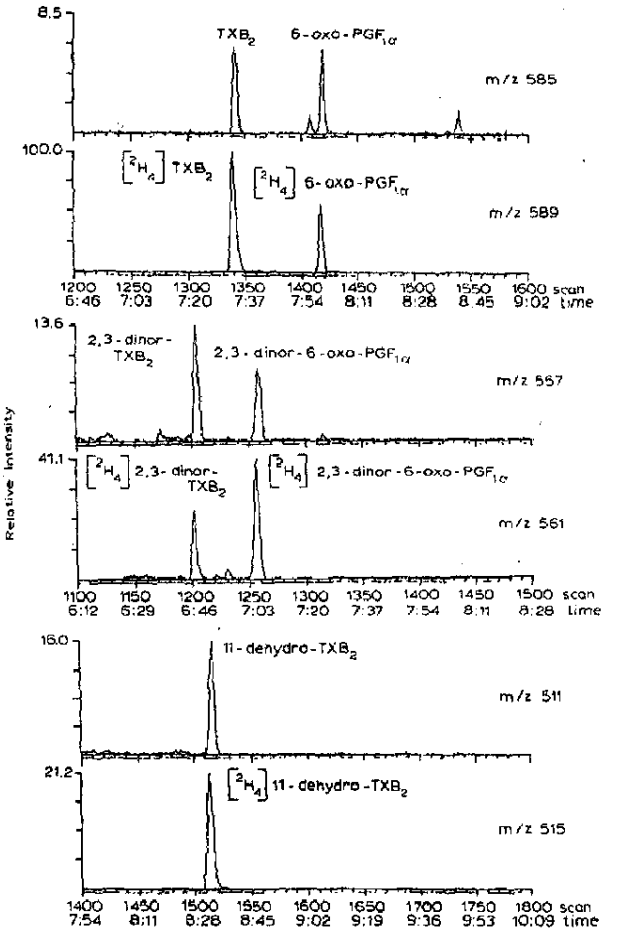

Fig. 1. Capillary column gas chromatography-mass spectroscopy. Selected ion monitoring chromatograms showing endogenous TXB_2, 6-oxo-$PGF_{1\alpha}$, 2,3-dinor-6-oxo-$PGF_{1\alpha}$, 2,3-dinor-TXB_2 and 11-dehydro-TXB_2 and their deuterated standards extracted from 10 ml urine.

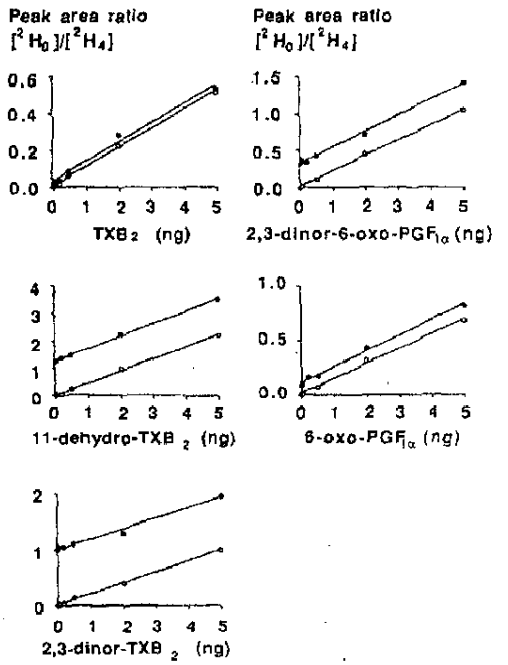

Fig. 2. Calibration curves generated for TXB_2, 2,3-dinor-TXB_2, 11-dehydro-TXB_2, 2,3-dinor-6-oxo-$PGF_{1\alpha}$ and 6-oxo-$PGF_{1\alpha}$, and their respective deuterated standards extracted from water (○) and from a pooled urine sample (●). Intercepts on the ordinates represent endogenous prostaglandins and thromboxanes in the urine.

retained through ammonium sulphate precipitation and dialysis. Residual TXB_2 was removed from the columns by subjecting them to a complete extraction and elution cycle before use in quantitative assays. Recovery of thromboxanes and prostaglandins through the immunoaffinity columns was consistently larger than 60% and was independent of the degree of cross-reactivity with antibody (indicating an excess of binding sites on the columns). There was no evidence of isotope discrimination in antibody binding. Calibration curves for standards extracted from buffer were comparable with those obtained using conventional extraction techniques (data not shown). Intra- and inter-assay coefficients of variation in eicosanoid determinations in samples taken from a common urine pool were less than 4 and 12%, respectively. The urinary prostaglandins and thromboxanes were stable at $-20\,^{\circ}C$ over a 6 month period.

The excretion rates of prostaglandins and thromboxanes by chronic smokers and non-smokers are shown in Fig. 3. There were significant elevations in the excretion rates of 2,3-dinor-TXB_2 ($2P < 0.001$), 11-dehydro-TXB_2 ($2P < 0.001$), 6-oxo-$PGF_{1\alpha}$ ($2P < 0.02$) and 2,3-dinor-6-oxo-$PGF_{1\alpha}$ ($2P < 0.02$). There was a significant positive correlation ($2P < 0.05$) between average reported cigarette consumption and both 2,3-dinor-TXB_2 and 11-dehydro-TXB_2 excretion (Fig. 4). The ordinate

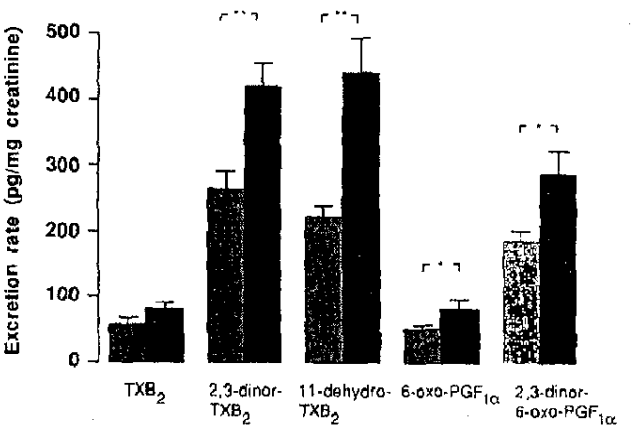

Fig. 3. Mean excretion rates (±S.E.M.) of prostaglandins and thromboxanes in 30 chronic smokers (solid bars) and in 37 non-smokers (cross-hatched bars). 24 h Urine collections were made and samples assayed by immunoaffinity chromatography and capillary column gas chromatography-electron capture negative ion chemical ionisation mass spectrometry. (** $2P < 0.001$; * $2P < 0.02$).

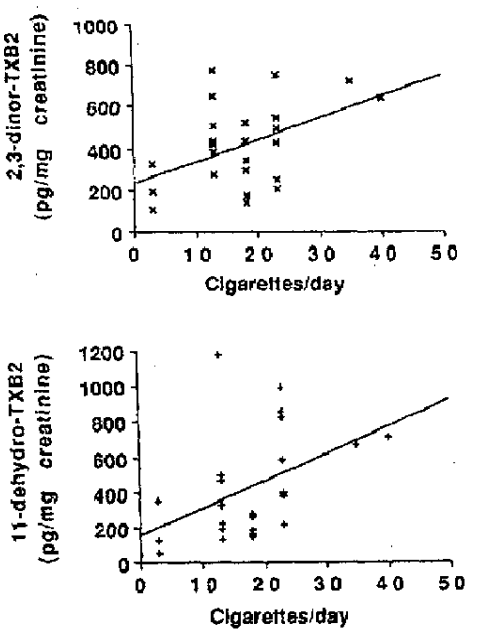

Fig. 4. Correlations ($n = 27$) between reported average daily cigarette consumption and urinary excretion of 2,3-dinor-TXB_2 ($r = 0.44$; $2P < 0.05$) and 11-dehydro-TXB_2 ($r = 0.45$; $2P < 0.05$).

intercepts of the linear regression lines were 233 and 151 pg/mg creatinine for 2,3-dinor-TXB_2 and 11-dehydro-TXB_2, respectively, and are comparable with the mean excretion rates of these metabolites in non-smokers. There was no significant difference between the rates of excretion of TXB_2 in the smoking and non-smoking groups. There was no correlation between eicosanoid excretion rate and urine pH.

There was no significant change in the excretion rates of PGI_2- or TXA_2-derived products following acute cigarette exposure. Plasma cotinine concentrations increased from a mean baseline of 0.4 ± 0.3 ng/ml to 4.7 ± 2.9 ng/ml 1 h after the smoking period. 21 hours later, the plasma concentration had decreased to a mean value of 3.4 ± 2.1 ng/ml. On control days, cotinine plasma concentrations at similar time points were 0.8 ± 0.2, 0.6 ± 0.2 and 0.4 ± 0.1 ng/ml, respectively.

Discussion

This study reports the first assay of urinary 11-dehydro-TXB_2 combined with TXB_2, 2,3-dinor-TXB_2, 6-oxo-$PGF_{1\alpha}$ and 2,3-dinor-6-oxo-$PGF_{1\alpha}$, using immunoaffinity chromatography and GC-ECMS. The method requires no extensive clean up procedures and up to 30 samples can be routinely processed in a single day and analysed by GC-ECMS in a further day. This methodology offers the prospect of evaluating pathological conditions where the balance between PGI_2 and TXA_2 may be altered in vivo. However, there is marked inter-individual variability in the rates of excretion of PGI_2 and TXA_2 products in urine [13,14,20]. Diurnal variations in excretion of 2,3-dinor-TXB_2 and 2,3-dinor-6-oxo-$PGF_{1\alpha}$ have been reported [20,21], and the excretion of the latter metabolite is increased with physical exercise [20]. It was necessary therefore to choose groups well matched for age, sex and physical activity to study any alterations in eicosanoid excretion that are caused by smoking.

Our observations in chronic cigarette smokers confirm previous reports that the urinary excretion of 2,3-dinor-TXB_2 is increased in smokers [13,14]. In this study, we have demonstrated that excretion of 11-dehydro-TXB_2 is also elevated in cigarette smokers, and by a similar magnitude to the increase in 2,3-dinor-TXB_2. This finding argues against a diversion of the metabolism of TXA_2 and favours increased biosynthesis. The increases observed in thromboxane metabolite excretion are significant ($2P < 0.001$), but the range of values in the smoking and non-smoking groups shows some overlap. However, there is a marked positive correlation between cigarette consumption and thromboxane metabolite excretion. An increase in average consumption of 10 cigarettes per day is associated with an increase in excretion of 101 pg/mg creatinine 2,3-dinor-TXB_2 and 154 pg/mg creatinine 11-dehydro-TXB_2. In contrast, TXB_2 in urine is thought to be derived primarily from the kidney [22] and was not significantly increased in smokers, nor was it correlated with cigarette consumption.

Increased TXA_2 formation in young, apparently healthy smokers, is potentially of considerable pathological importance, in view of the increased risk of thrombotic and pulmonary diseases in cigarette smokers and the potent biological effects of TXA_2 on platelets and vascular smooth muscle. Possible mechanisms for increased biosynthesis of TXA_2 are direct activation of platelets by reactive components in cigarette smoke or by adrenaline release from the adrenal gland by nicotine. These mechanisms seem unlikely because short-term exposure of normally non-smoking subjects to cigarette smoke did not increase the excretion of thromboxane metabolites. Nowak's study [14] favours the platelet as a source of increased TXA_2 biosynthesis in smokers, but whatever the origin of this increse, the effect requires habitual exposure to cigarette smoke. It is possible that increased TXA_2 biosynthesis is caused by indirect activation of platelets by atheromatous deposits on the walls of blood vessels. FitzGerald and co-workers [23,24] have reported increased excretion of 2,3-dinor-TXB_2, 11-dehydro-TXB_2 and 2,3-dinor-6-oxo-$PGF_{1\alpha}$ in patients with severe atheromatous disease. The increases in urinary excretion of both TXA_2- and PGI_2-derived products in the young soldiers may reflect

early signs of smoking-induced vascular damage. Alternatively, it is possible that subtle alterations in the cellular composition of the lungs of smokers, such as infiltration of neutrophils or macrophages which can synthesise TXA_2 [25–27] could account for the excess production of TXA_2.

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